Aus der Dermatologischen Universitätsklinik Basel
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METHOTREXATE AND IMMUNITY

INAUGURALDISSERTATION
zur Erlangung der Doktorwürde
der Medizinischen Fakultät der Universität Basel

Vorgelegt von
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J. A. Barth, Leipzig 1966

Von der Medizinischen Fakultät genehmigt
auf Antrag von Prof. Dr. R. Schuppli

Tag der Promotion: 11. März 1966

UNIV.
LEIDEN
BIBL.

Sonderdruck aus „Allergie und Asthma“ **12**, 309 (1966)

The basis of immunity is the interaction in the organism between an antigen and its specific antibody. This interaction can manifest itself in different ways. It can work advantageously (effet protecteur) or disadvantageously (effet pathogène); advantageously when it protects the organism from destruction (neutralization of an infective virus), disadvantageously when it causes destruction or disease (anaphylactic shock or autoimmune disease). Researchers in the last few years have tried to modify or check disadvantageous antigen-antibody reactions by removing either the antigen or the antibody or both. Lately a better understanding of antibody has offered new possibilities of preventing its production or attacking it.

In 1946 *Chase* found that he could abolish an antigen-antibody reaction – a delayed type of skin hypersensitivity to chemicals – by administering the chemical sensitizing agent either orally or systemically before exposing the sensitive organ (skin) to it. This he termed "immunologic unresponsiveness to orally administered haptens". *Felton* in 1949 described a condition in which mice were unable to produce antibody in response to large amounts of slowly metabolized polysaccharide antigens, and which he called "immunologic paralysis".

Billingham, Brent & *Medawar* (1953) brought about a state of "acquired immunologic tolerance" by administering large amounts of antigen to a newborn animal not yet capable of producing antibody. *Dixon* & *Maurer* (1955) overloaded an adult animal with huge amounts of antigen to a state of "immunologic unresponsiveness". They also infused large amounts of protein antigen to X-irradiated adult animals to produce a state of "radiation-induced unresponsiveness". *Schwartz* & *Dameshek* (1959) described a state of "druginduced immunologic tolerance", brought about by administration of large quantities of antigen to mature animals previously treated with cytotoxic drugs.

Interest in immunologic tolerance – the loss of the ability to react to repeated exposure to an antigen *(Schwartz* [1965]) – developed when the clinician envisaged the possibility of using drugs to prevent and treat clinically undesirable immune responses. Many drugs were then tested within a short period of time for an ability to inhibit the immune response by suppressing antibody production. The amount of antibody produced was determined from a number of different in vitro antigen-antibody reactions or from an in vivo immunologic reaction. The in vivo reactions were found to be either immediate (réaction immédiate) or delayed (réaction tardive). The immediate type is either generalized (anaphylactic reaction) or localized (Arthus phenomenon) and is dependent on circulating (humoral) antibody and nonspecific inflammatory cells. The delayed reaction is dependent on cell-bound (sessile) antibody and specific inflammatory cells.

One of the drugs found to be capable of suppressing both types of antibody was amethopterin (Methotrexate, Lederle Laboratories). Methotrexate (MTX) is a folic acid antagonist that interferes competitively with the conversion of folic acid to folinic acid by inhibiting folic acid reductase, the enzyme necessary for this con-

version. Folinic acid is necessary for the production of nucleic acids and some proteins as it is the carrier of one-carbon moieties involved in the formation of purines, pyrimidines and some amino acids. Nucleic acids are indispensable for cell division. The exact role of cell division in antibody production has not been completely clarified. There is, however, strong evidence that antibody-forming cells – plasma cells in the case of humoral, and immunologically committed small lymphocytes in the case of cellular antibodies – divide from pyroninophilic cells (hemocytoblasts) which arise from uncommitted small lymphocytes (precursor cells) on interaction with antigen *(Gowans* & *McGregor* [1965]). *Turk* & *Stone* (1963) concluded from their observations that MTX appears to block the development of immunologically committed small lymphocytes and plasma cells from hemocytoblasts, a cell multiplication process.

Haas et al. (1957a, 1957b), *Lerner* & *Haas* (1958) and *Briggs* (1959) noticed that the lymphocytic reaction and related immune response to lymphocytic choriomeningitis was insufficient to produce histopathological lesions in animals deficient in folic acid. *Oleson* (1950), *Ludovici* & *Axelrod* (1951), *Wertman* et al. (1952) and *Sugai* (1958) demonstrated a suppression of humoral antibody production in folic acid-deficient states.

Using sheep red blood cells as antigen and mice as experimental animals, *Malmgren, Bennison* & *McKinley* (1952) and *Brown* & *Berenbaum* (1964) showed that a sufficient dose of MTX given at the right time inhibited humoral antibody production. *Nathan* et al. (1961), who also used sheep red blood cells and mice, obtained positive results, although suppression of antibody titer was only moderate. *Calne* & *Leibowitz* (1963) were able to inhibit the hemolysin titer against sheep red blood cells with MTX in guinea pigs. *Santos* & *Owens* (1962, 1963, 1964) showed the action of MTX to be statistically significant in suppressing production of agglutinin against sheep erythrocytes in rats. *Brooke* (1960), however, found that rabbits produced circulating antibody against sheep erythrocytes and bovine gamma globulin as normal when given MTX in an attempt to suppress this response.

Berenbaum (1962a, 1962b) and *Berenbaum* & *Brown* (1964, 1965) gave typhoid-paratyphoid A and B vaccine to mice and found that the immune response was only sensitive to MTX when the latter was injected one to five days after the antigen and that maximum suppression occurred when it was injected on the second day. *Berenbaum* & *Brown* (1965) also found that folic acid, given eight hours or more after MTX, inhibited its toxic effects without affecting suppression of the immune response.

Thomas et al. (1962, 1963a) investigated the production of free antibody against attenuated distemper virus in dogs, and found it to be completely suppressed when MTX was given. *Vedovini* et al. (1963) observed that MTX reduced production of antibody against influenza virus in mice. This effect of MTX was accentuated by a diet deficient in folic acid. Similar results were obtained by *Friedman, Baron* & *Buckler* (1962) with vaccine virus in guinea pigs, using a combination of X-irradiation and MTX. *Šterzl* (1961) showed that aminopterin – a folic acid antagonist similar to amethopterin (MTX) – suppressed antibody production at the cellular level: no antibody was produced when a bacterial antigen was injected into neonatal rabbits to which adult spleen cells (containing many small lymphocytes) had been transferred.

The influence of MTX on cell-bound antibody production was reported by *Friedman* (1964) and *Friedman* et al. (1961, 1963a, 1963b), when they showed both the active and passive tuberculin reactions in guinea pigs to be inhibited after

tubercle vaccination and lymph node cell transfer respectively. *Floersheim* (1965) in a comparative study also found MTX to inhibit the tuberculin reaction, although not to as great an extent as some of the other drugs he tested. *Prichard* & *Hayes* (1961) demonstrated suppression of the immunologic response of guinea pigs to tubercle bacilli by aminopterin; there was no evidence of cutaneous tuberculin hypersensitivity and, histologically, caseation necrosis occurred. In the same way, *Heierli* (1966) suppressed the trichophytin reaction with MTX.

Working on chemical contact hypersensitivity – a delayed type of antibody reaction – *Turk* (1964) found he could prevent the development of sensitization with MTX. *Page* (1964) showed that MTX did not inhibit the lymphocytic response to inflammation and that *Turk*'s findings applied rather specifically to the building of antibodies.

Experiments were also carried out with folic acid antagonists to suppress the immune reaction in autoimmune disease and in tissue and cell transplantations. Exactly what type of antibody is involved here still remains to be elucidated.

In autoimmune diseases some authors suggest serum antibodies as the basic cause of the pathological lesions *(Roitt* et al. [1962]), others suggest cellular antibodies *(Rose* et al. [1962]), and others again contend that both are involved *(Mackay* & *Burnet* [1963]). *Schwartz* (1965) suggests possible inflammatory reactions secondary to an immunologic response. It is possible to understand the discrepancy when experimental evidence suggests that delayed hypersensitivity and humoral antibody reaction are parts of the same fundamental process *(Salvin* & *Smith* [1960], and *Karush* & *Eisen* [1962]). For all practical purposes it should be sufficient to know the action of an immune blocker on the component parts to know its action on the whole. As the above review shows, MTX tends to block both humoral and cellular responses, and it can be expected to act similarly in a disease that might involve both of these processes. *Spiegelberg* & *Miescher* (1963) analyzed both parts and the whole of experimental immune thyroiditis – an experimental autoimmune disease – in guinea pigs. They found that aminopterin suppressed humoral antibody production as well as delayed hypersensitivity to thyroglobulin and that immune thyroiditis did not appear as long as the drug was given.

Brandriss (1963), *Goodman* et al. (1963), *Calne* & *Leibowitz* (1963), and *Brandriss, Smith* & *Friedman* (1965), suggesting allergic encephalomyelitis in guinea pigs as a good experimental model for autoimmune diseases, tested MTX as a drug for treatment and prevention and found it to be very effective. *Gubner* et al. (1951) and *Black* et al. (1964) reported on the use of folic acid antagonists in the treatment of rheumatoid and psoriatic arthritis in man. Psoriatic arthritis was substantially improved, but the effect on rheumatoid arthritis was only subjective. It is surprising how few reports have been made on the influence of MTX on autoimmune diseases.

Voisin (1964) recently reviewed the relation and similarity between autoimmunity and transplantation immunity. There have been many reports on transplantation tolerance and most authors are of the opinion that both humoral and cell-bound antibodies are concerned in transplantation reactions *(Gorer* [1960]; *Hellström* & *Möller* [1965]).

Most of the transplantation experiments with MTX reported have been skin homotransplantations. Most authors obtained positive results in suppression of the immune reaction (graft take). Prolonged graft take was achieved in guinea pigs *(Berenbaum* [1963, 1964b]) and toxic effects from the drug could be alleviated by the subsequent administration of folinic acid *(Berenbaum* [1964b]). In rats *(Santos*

& *Owens* [1965]) prolonged graft survival was only apparent when MTX was given in multiple doses from five to seven days after transplantation. In mice (*Glynn* et al. [1963] and *Floersheim* [1965]) skin graft survival was shown to be prolonged when MTX was given after transplantation. The survival time was even greater when *Medawar* (1963) and *Uphoff* & *Pitkin* (1962) applied the *Dixon* & *Maurer* (1955) state of "immunologic unresponsiveness" together with "drug-induced immunologic tolerance": they used tissue extract to weaken the immunologic reaction as well as giving MTX. *Skowron* (1964) went a step further. He overloaded mice with antigen to a state of "immunologic unresponsiveness" by coelomic parabiosis and maintained this abdominal junction as well as hybrid skin grafts with MTX. *Hechtman* et al. (1962) showed that continuous MTX treatment could suppress skin graft rejections in dogs. *Epstein* et al. (1965) then added "immunologic unresponsiveness" by setting up cross-circulation between the donor and the host dog – antigen overload – and prolonged skin graft survival with MTX. Interesting and constant results among the different workers have been reported with rabbits. *Brook* (1960), *Meeker* et al. (1960) and *Flamm* et al. (1962) found that rejection of skin transplants could not be suppressed by folic acid antagonists. Negative results have also been obtained with organ transplantations in dogs. *Alexandre* & *Murray* (1962) and *Zukoski* et al. (1962) were unable to prevent rejection of kidney grafts in dogs with MTX. *Reemtsma* et al. (1962) and *Blumenstock* et al. (1963) succeeded, however, in prolonging canine heart transplantation with a folic acid antagonist, and *Blumenstock* et al. (1961, 1962a, 1962b, 1962c) and *Gago* et al. (1964) maintained functioning lung homografts in dogs using various methods in conjunction with MTX.

Tumor transplantation was also tried, successful transplantation being judged by tumor growth and subsequent death of the host when the immune graft response was suppressed. *Glynn* et al. (1963) and *Humphreys* et al. (1961, 1962, 1963) demonstrated this to occur in mice when MTX was given after leukemia tumor cell injections. *Uphoff* (1961) showed the same to be true when viable cells (antigen) and MTX were injected before the tumor cells.

Another step in transplantation was prior X-irradiation: graft take was very good, but the animal would succumb to some natural disease that it would have otherwise withstood. It was then found that if allogenetic marrow was engrafted after lethal irradiation the animal withstood the infection, but succumbed to a "homologous secondary disease", which is thought to be the result of an immunologic reaction of the donor cells against the homologous host. *Lochte* et al. (1962a, 1962b) and *Schwartz* & *Beldotti* (1965) showed that this foreign marrow reaction or "homologous disease" could be prevented if MTX was given shortly after the marrow graft. *Mathé* et al. (1962) obtained similar results with MTX given after lethal X-irradiation to treat leukemia. *Uphoff* (1958) applied this to transplantations, successfully transplanting skin homografts after lethal irradiation, homologous marrow grafting and suppression of homologous disease with MTX. *Woodruff* (1963), using mice, and *Thomas* et al. (1962b, 1962c, 1963b), using dogs, found this successful to prolong skin homografts. *Blumenstock* et al. (1962b) prolonged lung homotransplantation time in his dogs by this means.

MTX also has a significant influence on infections. In animals, the course of experimental infections was found to have been affected by treatment with MTX, most probably as the result of an altered immune status. *Thomas* et al. (1962a, 1963a) reported that this altered immune status caused dogs to succumb to fatal illness and viremia when treated with MTX after being inoculated with a benign

attenuated distemper virus. *Friedman, Baron & Buckler* (1962) found, however, that their irradiated guinea pigs were able to eliminate vaccinia virus when treated with MTX, although no antibody was produced. Whether pre-irradiation is in fact necessary for virus elimination when the immune response is blocked remains to be seen. Mice infected with lymphocytic choriomeningitis virus and then treated with MTX were spared, but were not able to eradicate the infection and late post-inoculatory viremia, although this was found dependent on the dosage of MTX *(Haas* et al. [1956, 1957b]; *Lerner & Haas* [1958]; *Hotchin* [1962]). *Potter & Haas* (1959) in an interesting experiment found that MTX not only impeded the immune homograft reaction, but also the immune virus reaction. This they demonstrated by transplanting a tumor infected with lymphocytic choriomeningitis virus to a host already immune to the virus under treatment with MTX. The tumor grew, i.e. the homograft reaction was suppressed, and when later transplanted to a non-immune host it still showed infectivity, demonstrating that the immunity in the first host was suppressed sufficiently to permit infectivity to remain.

Suppression of the immune response has also been found to alter the course of tubercle *(Prichard & Hayes* [1961]) and fungus infections *(Heierli* [1966]).

Most of these authors have found in their own way that the time, amount and method of drug administration vary according to the antigen and animal. Many have come to the conclusion that the opportune time is approximately two days after antigen administration, although some discrepancies exist. Histologically this moment does, however, correspond to the increased cell division noted by *Gunderson* et al. (1962) in the rat spleen stimulated with antigen and by *O'Brien* and *Coons* (1963) in their tissue cultures. It also seems logical when one recalls that folic acid antagonists prevent cell division by inhibiting the production of nucleic acids by blocking purine and pyrimidine synthesis. However, because of the differences in dosage, dosage schedule, sensitization methods, antigens and analytic methods, collective statements are still somewhat premature. To try to narrow down these variables, the following experiment was undertaken:

Material and methods

Animals: As experimental animals twenty randomly bred guinea pigs weighing 200–400 g were used. They received a diet of turnips, hay and bread ad libitum.

Antigen: As antigen a filtered 10% solution of egg-white in saline was used. Between injections it was stored at + 2° C. 2 ml were injected i.p. four times in one week.

Antibody: Blood was obtained by cardiac puncture on the 28th experimental day (20 days after the beginning of the sensitization period). It was allowed to clot and retract for two hours at room temperature and then centrifuged for 20 minutes at 3000 rpm. The amount of antibody in the serum was determined by a microgel diffusion method. Agar gel was prepared in a 1% concentration. Pure agar (Behring-Werke, Marburg a.d. Lahn) was dissolved by heating in a solution made up of 93% saline, 5% borate buffer p_H 8.0, 1% merthiolate (0.1% concentration in saline) and 1% trypan blue (1% in aqueous solution). 2.5 ml were pipetted onto microscope slides and allowed to harden. Six wells equidistant from each other and from a center well were cut with a 5 mm cork borer and the surplus agar extracted with a water suction pump. Two of the forms thus produced were set onto each slide and the experiments carried out in duplicate. The outside wells were filled with dilutions of antigen varying in geometric progression, the concentration in each successive well being half of that in the previous one. The first contained a 10% egg-white solution (1 : 1), progressively diluted down to 1 : 32 in the last. The serum was placed in the center well. The precipitation was evaluated from negative (–) to + 6 according to the number of wells showing a precipitation. Negative (–) signified no precipitation. The slides were kept humid at a temperature of + 2° C for 48 hours before being examined.

Cytostatic: Methotrexate (Amethopterin, A-amino-10-methyl-pterolglutamic acid; Lederle Laboratories) was dissolved in distilled water to a concentration of 10 mg per ml immediately before using. 1.5 ml of the solution was injected intraperitoneally every fourth day.

Procedure: The guinea pigs were randomly separated into 4 groups of 5. The experiment lasted 30 days.

Group I received MTX i.p. on days 1, 5, 9, 13 and 17.
Group II received MTX i.p. on days 9, 13, 17 and 21.
Group III received MTX i.p. on days 17 and 21.
Group IV received no MTX and served as control.
All animals received 2 ml of a 10% egg-white solution i.p. on days 9, 11, 14 and 16.

Cardiac puncture was done on day 28. The serum was tested for antibody a few hours later by a microgel diffusion method and examined after a further 48 hours on day 30.

Results

The results and procedure are simplified in Table 1.

Serum from group I animals, receiving 5 injections of MTX before and during the period of sensitizing injections, showed that 2 of 4 animals were not sensitized. The 5th guinea pig (no. 067) died on day 22 and its serum was collected, frozen and tested together with the others on day 28. The evaluation of this serum is left to the reader, as the day of death could be critically regarded as borderline as far as sensitization time is concerned.

Serum from group II animals, receiving 4 injections of MTX during and after the week of sensitizing injections, showed that 3 of 5 serums contained no antibody to egg-white.

Group III animals, receiving 2 injections of MTX after the week of sensitizing injections, produced no antibody at all.

Serum from group IV animals, receiving no MTX and only sensitizing injections of egg-white, were all positive for antibody, although varying from + 1 to + 5.

In the following weeks 9 animals died, 8 of which had had negative serums.

Discussion and conclusions

When one compares the animals that received MTX (groups I, II and III), regardless of the dosage times, with those that did not, it is seen that MTX inhibited antibody production in two-thirds of the cases. When one goes a little further and examines the dosage schedule group by group, it can be seen that administration of MTX before and during the period of sensitizing antigen injections (group I) has no advantage over administration during and after the injections (group II). Furthermore, when MTX is given after the antigen (group III) no antibody is produced as compared with controls (group IV). It is interesting, however, that group II animals, also receiving MTX after the week of sensitization, did not all present negative serums. The possibility of a neutralizing or adjuvant effect of long or premature administration on antibody production must be considered here. Unexplained variability in the immune suppressive effects of MTX has been reported by *Berenbaum* (1963), *Brown* (1964) and *Hechtman* et al. (1962). An adjuvant effect of other immune suppressive drugs has also been observed, mainly when the drug was given before the antigen (*Buskirk* et al. [1963] and *Merritt* & *Johnson* [1963]). *Dixon* & *McConahey* (1963) have reported the same effect with X-irradiation. *Werkheiser* (1963) tried to organize the facts of this phenomenon and suggested tissue permeability as the relevant factor. *Jacobson* & *Cathie* (1960) claimed that some cells can inactivate folic acid inhibitors. *Bertino* (1963) and *Bertino* et al. (1962) suggested that the neutralizing or adjuvant effect is due to synthesis of folic acid reductase – the enzyme inhibited by MTX –, which can increase proportionate to exposure to MTX. An interesting explanation devised by *Talmage* & *Perlman* (1963) is that complement-blocking polynucleotides are released as a result of cytolytic effects. Complement, which they assume to suppress

Table 1

Group	Experimental Day																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
067	*M*				*M*				*ME*		E		*M*	E		E	*M*													—
073	*M*				*M*				*ME*		E		*M*	E		E	*M*													—
I 074	*M*				*M*				*ME*		E		*M*	E		E	*M*													+4
077	*M*				*M*				*ME*		E		*M*	E		E	*M*													+5
088	*M*				*M*				*ME*		E		*M*	E		E	*M*													—
065									*ME*		E		*M*	E		E	*M*				*M*									+4
069									*ME*		E		*M*	E		E	*M*				*M*									—
II 075									*ME*		E		*M*	E		E	*M*				*M*									—
076									*ME*		E		*M*	E		E	*M*				*M*									—
082									*ME*		E		*M*	E		E	*M*				*M*									+4
063									E		E			E		E	*M*				*M*									—
064									E		E			E		E	*M*				*M*									—
III 066									E		E			E		E	*M*				*M*									—
068									E		E			E		E	*M*				*M*									—
070									E		E			E		E	*M*				*M*									—
071									E		E			E		E														+5
072									E		E			E		E														+1
IV 078									E		E			E		E														+5
079									E		E			E		E														+1
081									E		E			E		E														+5

M = Methotrexate 15 mg E = egg-white solution 10%, 2 ml — = no precipitation + 1 to + 8 = positive precipitation

the division of antibody-forming cells, then becomes blocked and an increase in antibody production could occur. The precise explanation for the neutralizing or adjuvant effect remains to be elucidated. This effect is, however, the probable explanation of why MTX does not act as effectively when given before or during antigen administration (groups I and II) as when given after (group III). This need not exclude a specific effect of MTX on dividing pyroninophilic cells stimulated to high metabolic activity by antigens.

Nine animals died for different reasons, but since eight of them had had demonstrable suppression of antibody production, some deaths could probably be attributed to a lack of resistance to an infective agent.

Some of the characteristics of MTX in immune response that can be gleaned from this and other experiments are that folinic acid can alleviate its toxic effects without affecting the suppressed immune response *(Berenbaum* [1964a]); that MTX-induced immunologic tolerance is specific to a given antigen *(Santos* & *Owens* [1963]; *Uphoff* & *Pitkin* [1962]) when the antigen prevails within the therapeutic period of the drug; and that once induced, this immunologic tolerance can be terminated by discontinuation of the drug *(Spiegelberg* & *Miescher* [1963]; *Thomas* et al. [1962a]; *Friedman* [1964]; *Friedman* & *Buckler* [1963a]), immunization with a cross-reactive antigen *(Salven* & *Smith* [1964]) or administration of desoxyribonucleic acid *(Feldman* et al. [1962]).

The termination of established immunity by means of MTX is still inconclusive. Various degrees of depression have nevertheless been reported *(Thomas* et al. [1962a]; *Butler* [1961]; *Friedman* [1964]; *Goldin* et al. [1962]). *Humphreys* et al. (1961, 1962) found, however, that once immunity to a homograft leukemia tumor had been established, it could not be suppressed with MTX.

It can be concluded that MTX obviously has a place in the prevention and treatment of disadvantageous antigen-antibody reactions by suppressing antibody production, provided the proper dosage and dosage schedule is maintained.

Summary: The literature dealing with methotrexate and the suppression of the immune response, as well as some theories about it are reviewed. An experiment to determine the therapeutic period was carried out using guinea pigs sensitized within the course of one week with multiple doses of protein antigen. The conclusions drawn are that the therapeutic period follows the week of antigen injections and that previous treatment with methotrexate has no advantages, possibly as a result of an adjuvant or neutralizing effect on antibody production of long or premature administration of methotrexate.

Zusammenfassung: Es wird die Literatur über die immunsuppressive Wirkung von Methotrexat besprochen. An Meerschweinchen, die mit verschiedenen Dosen von Eiweiß sensibilisiert wurden, wurde die Zeit bestimmt, in der diese Wirkung eintritt. Es ergab sich, daß sie eine Woche nach der Antigeninjektion optimal ist und daß eine Vorbehandlung mit Methotrexat keinen Vorteil hat.

Acknowledgement. I should like to express my very sincere gratitude to Miss Doris Frank for her willing assistance, and to Mr. Brian Levin for his painstaking efforts to ensure clarity and conciseness in the text.

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Received 3. 7. 66
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